

The different types of sperm morphology and behavior within a single species

Why do sperm of squid sneaker males form a cluster?

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Some coastal squids exhibit male dimorphism (large and small body size) that is linked to mating behaviors. Large “consort” males compete with other, rival males to copulate with a female, and thereby transfer their spermatophores to her internal site around the oviduct. Small “sneaker” males rush to a single female or copulating pair and transfer spermatophores to her external body surface around the seminal receptacle near the mouth. We previously found that in *Loligo bleekeri*, sneaker sperm are ~50% longer than consort sperm, and only the sneaker sperm, once ejaculated from the spermatophore (sperm mass), form a cluster because of chemoattraction toward their own respiratory CO₂. Here, we report that sperm clusters are able to move en masse. Because a fraction of ejaculated sperm from a sneaker’s spermatophore are eventually located in the female’s seminal receptacle, we hypothesize that sperm clustering facilitates collective migration to the seminal receptacle or an egg micropyle. Sperm clustering is regarded as a cooperative behavior that may have evolved by sperm competition and/or physical and physiological constraints imposed by male mating tactics.

Sperm are cells that are highly specialized to fertilize an egg. Therefore, in each species, sperm are selected for optimum morphology and function, and as a result, sperm populations generally become homogeneous within species. Meanwhile, alternative mating tactics,¹ usually found in male dimorphism, can make a difference in sperm competition risk, which might have an impact on sperm morphological and functional traits. Although the theory predicts that the sperm size can be differently determined in relation to sperm competitiveness within species, empirical studies have failed to find good examples. We recently found, however, that males of the squid *Loligo bleekeri* produce 2 differently sized spermatozoa with fertilizing capacity.² Small sneaker males produce ~50% longer spermatozoa than large consort males (average total length of ~73 µm in consorts and ~99 µm in sneakers).² No significant difference was found in the swimming speed between sneaker and consort spermatozoa.² An in vitro fertilization assay with video microscopy revealed that both consort and sneaker sperm are capable of accumulating around egg micropyle and thereafter passing through the micropore to fuse with the egg (data not shown). These fertilized eggs have developed normally, indicating that they are eusperm. Furthermore, mitochondrial DNA analysis found no bias in the haplotype frequency, thus ruling out the possibility of cryptic species.²

Females of this species are promiscuous, allowing copulations by both types of males sequentially or even simultaneously, which results in a high frequency of multiple paternity in single egg clutches (egg strings).³ Although sperm competition theory predicts that sneaker males produce relatively larger number of sperm than consort males to compensate disadvantage in mating behavior, the number of spermatozoa within a single spermatophore were ~5-fold greater in consort males than sneaker males (consort: 15×10^{10} cells, sneaker 3×10^{10} cells).² Because sperm storage sites for sneaker or consort sperm are located outside or inside the female’s body, respectively, the sperm–egg encounter may occur in different places and at different times. Thus, although paternity is mixed, evidence for existence of sperm competition between sneaker and consort remains elusive.

We recently found that sneaker sperm, but not consort sperm, exhibit clustering (swarming) behavior when ejaculated from spermatophores (sperm masses) (Fig. 1).⁴ This clustering is mediated by sperm chemotaxis to CO₂ emitted during their respiration. We investigated the molecular pathway of CO₂ detection by sneaker sperm, and found that catalytic activity of membrane-bound carbonic anhydrase (CA) and a concomitant decrease in cytoplasmic pH (acidosis) are essential for sperm clustering.⁴ Proteome analysis in conjunction with transcriptome analysis confirmed the previously identified CA as a sole gene product in

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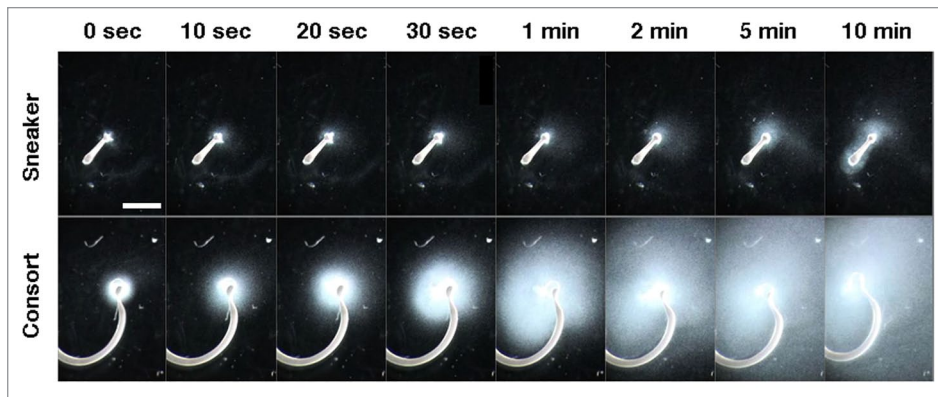


Figure 1. Time course of sperm ejaculation. Ejaculated consort sperm quickly diffuse away from the sperm mass, but sneaker sperm remain in the vicinity of the duct.

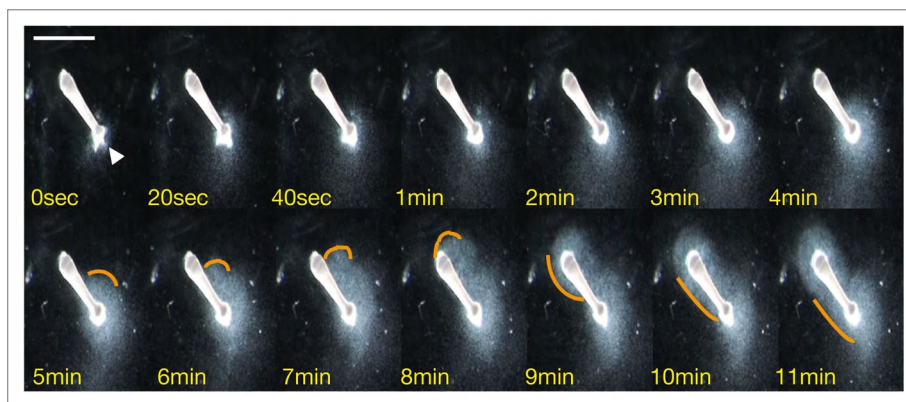


Figure 2. Ejaculation, clustering, and migration of sneaker sperm. Upon ejaculation, sneaker sperm form clusters and stay around the sperm mass. Time-lapse images reveal development of sperm clusters alongside the sperm mass. An orange line indicates the leading edge of a cluster. Arrowhead indicates a sperm ejaculatory duct. Bar: 2 mm.

sperm, suggesting that cytoplasmic CAs are absent, and therefore sensing of CO_2 occurs at the sperm surface (data not shown). Notably, consort sperm do also express functional CA, but proton transport across the plasma membrane would occur only in sneaker sperm. The transport molecule remains to be identified.⁴

Why do sneaker sperm form a cluster? We recorded the fate of sperm clusters that were formed around an ejaculatory duct of the sperm mass that was placed in a culture dish. We found that sperm clusters extended slowly, surrounding the sperm mass and remained in its close vicinity (Fig. 2). This phenomenon may suggest that the sperm mass also attracts single or clustered sperm cells by releasing CO_2 or other chemicals. We next performed a capillary assay to examine if sperm clusters are capable of moving en masse. Capillary-loaded sperm first appeared to be evenly distributed, but became concentrated in a regular, striped pattern with time (Fig. 3). This pattern was maintained for a certain period of time, and thereafter 2 neighboring strips came

closer to merge into 1 (Fig. 3). This suggests that sperm clusters are mutually attracted and move en masse, presumably due to a decreasing chemical gradient between the stripes.

In natural mating, sneaker males attach their spermatophores to the female's outer skin near the seminal receptacle where ejaculated sneaker sperm are stored. How ejaculated sperm move into the seminal receptacle is unknown.⁴ However, it would be conceivable to imagine that sperm clusters are capable of migrating along a gradient of chemical(s) released from a female's skin or the seminal receptacle.⁵

In the wood mouse, clustering of sperm enhances the forward movement that presumably facilitates transport through the oviduct.⁶ Cooperative behavior by sibling spermatozoa could be explained in the context of kin selection. Strong sperm competition could promote evolution of cooperative behavior among sibling sperm. Because sperm of diploid species share half their genes with siblings, it is more beneficial to help fertilization of siblings than of nonsiblings. Immediate clustering of ejaculates would be a means to increase successful cooperation among siblings. In squids, sperm clusters can form in nonsibling sneakers under experimental conditions (data not shown). However, because of immediate clustering, this is unlikely to occur in

physiological situations. In *Drosophila*, particular males often tend to dominate fertilization, a process known as sperm precedence. Sperm precedence is influenced by the male mating order. Sperm from the last male can replace sperm from previous males within the spermathecae.⁷ Occupancy within the limited sperm storage space would determine the paternity rate in *L. bleekeri*. Thus, clustering of sibling sperm followed by collective migration to the seminal receptacle may effectively dominate fertilization success (Fig. 4, left). It is also possible that sperm clusters remain around the egg periphery until a single sperm enters into the egg micropore, the channel below the micropyle funnel, at the time of fertilization. Sperm clusters would be able to move into and/or take up space in the micropyle funnel, thereby outcompeting nonsiblings (Fig. 4, right). Elucidating physiological role(s) for sperm clustering and understanding molecular mechanisms will provide insights into sperm cooperation under competitive and ecological environments.

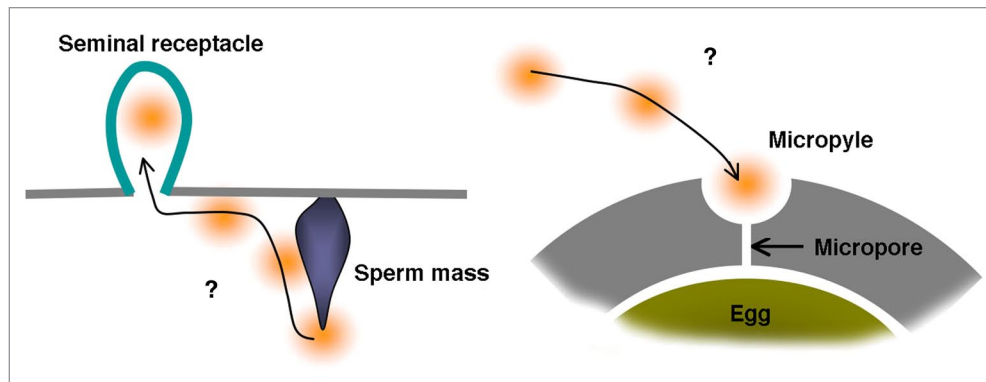


Figure 4. Possible roles of sperm clustering during postmating events in squid. Left: ejaculated spermatozoa from a sneaker male's sperm mass located externally on the female's skin migrate together to the seminal receptacle. Right: spermatozoa released from the sperm mass or the seminal receptacle migrate together to the egg's micropyle.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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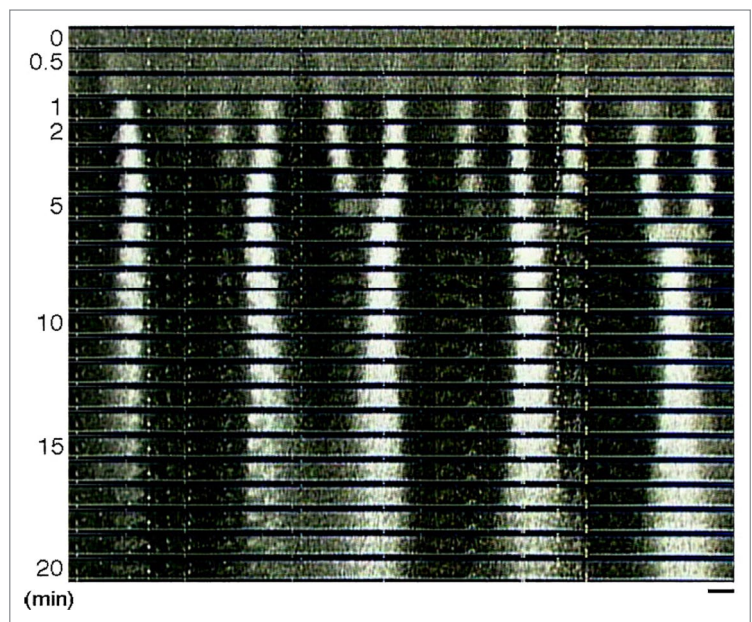


Figure 3. In vitro sperm clustering within a capillary tube. Sneaker sperm loaded into a glass capillary show time-dependent cluster formation. Uniformly distributed sperm cells ($t = 0$ min) become concentrated, appearing as fine stripes (average distance of ~ 0.53 mm between stripes, $t = 1$ min), followed by fusion of 2 adjacent stripes ($t = 6$ min) to form more stable clusters. The tight clusters loosen after a few minutes, presumably because the sperm become desensitized or a chemical gradient becomes smaller. Small clusters (stripes) tend to move toward large, neighboring clusters. Bar: 200 μ m.

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